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A strategy to make constitutively active MAP kinase by fusing with constitutively active MAP kinase kinase

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Abstract

Classical mitogen-activated protein kinases (MAPKs) play a pivotal role in a variety of cellular signal transduction pathways. MAPKs are activated by phosphorylation at specific threonine and tyrosine residues catalyzed by upstream MAPK kinases (MAPKKs). Mutations of these two activation phosphorylation sites into acidic amino acids, however, do not convert MAPKs into constitutively active forms. Here, we report an approach to make a molecule with constitutive MAPK activity. The nuclear export signal-disrupted, constitutively active MAPKK was fused to the N-terminal end of wild-type MAPK. When the resulting fusion protein was expressed in *Escherichia coli*, the MAPK moiety became phosphorylated and the fusion protein was constitutively active as MAPK. Moreover, when expressed in mammalian cultured cells, the fusion protein was also activated as MAPK and was able to induce marked morphological changes in NIH-3T3 cells. These results suggest that the fusion protein can work as constitutively active MAPK and that this approach may be applicable to other members of the MAPK family to make constitutively active forms.

Keywords: Extracellular signal-regulated kinase; Fusion protein; Mitogen-activated protein kinase cascade; Signal transduction

1. Introduction

Various extracellular stimuli evoke cellular responses via activation of protein kinase cascades [1,2]. Mitogen-activated protein kinases (MAPKs), also known as the extracellular signal-regulated kinases (ERKs), are serine/threonine protein kinases that play pivotal roles in transmitting various cellular signals to the nucleus. MAPKs can be activated directly by dual phosphorylation at threonine and tyrosine residues in the activation loop by upstream dual specificity protein kinases, MAPK kinases (MAPKKs) [3–10].

Constitutively active forms of proteins can be effectively used to analyze the physiological function of the molecules. Many proteins that are activated by phosphorylation have been reported to be artificially modified into constitutively active forms when an amino acid(s) at the activating phosphorylation site(s) is mutated to a phosphoamino acid-mimicking

Abbreviations: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; NES, nuclear export signal; GST, glutathione-*S*-transferase; GFP, green fluorescent protein; HA, hemagglutinin; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue

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acidic amino acid(s). Phosphorylation of two adjacent Ser/Thr residues in the activation loop of MAPKK (also known as MEK) is essential for its activation and in fact, alteration of these residues to acidic amino acids makes MAPKK constitutively active [11,12]. Another prevailing way to produce constitutively active proteins is deleting an inhibitory domain from a full-length protein. This strategy is applicable for proteins that possess a domain that regulates the activity negatively. As a typical example, Raf protein kinase possesses an inhibitory region at its N-terminus and deletion of this region renders Raf constitutively active [13].

Although MAPKs are known to be activated by phosphorylation at the conserved TXY motif in the activation loop [3–10], mutations of these phosphorylatable threonine and tyrosine residues to acidic amino acids fail to convert MAPKs into constitutively active forms. In addition, MAPKs are not known to have negative regulatory domains, thus, it is not easy to make MAPK constitutively active by deleting some portion of the molecules. It is important and useful to produce constitutively active forms of MAPKs not only for analyzing functions of MAPKs in a variety of biological processes but also for screening of drugs that inhibit the activated MAPKs. Although a gain of function mutation of a *Drosophila* MAPK homologue *rolled* was identified [14], the kinase activity of the mutant was very low [14,15] and the cause of the gain of function still remains to be elucidated.

The intracellular distribution of MAPK is regulated by its association with MAPKK [16]. Recently, conserved nuclear export signals (NESs) have been identified in many cytoplasmic proteins [17–19]. In the N-terminal region, MAPKK has a NES [20] that is responsible for cytoplasmic localization of not only MAPKK itself but also MAPK [16]. Moreover, the association between MAPKK and MAPK may enhance the efficiency of signal transduction from MAPKK to MAPK and assure its specificity [21].

In this study, we report an approach to produce a constitutively active form of MAPK. The idea is that MAPK must be efficiently phosphorylated and activated by MAPKK when MAPK locates adjacent to constitutively active MAPKK. In addition, the NES of MAPKK was disrupted to ensure that the fusion protein resides within the nucleus to have biological effects. Recently, Cobb and colleagues reported an achievement of constitutively active MAPK by making a fusion protein between nuclear localizable MEK1 and ERK2 [22].

2. Materials and methods

2.1. Plasmids, proteins, reagents and cell lines

cDNAs of *Xenopus* MAPK (wild-type and mutants) and MAPKK (wild-type and mutants) were described previously [16,20,23]. pGEX-6P for glutathione-*S*-transferase (GST) fusion proteins and PreScission protease were from Pharmacia. pEGFP-N1 for green fluorescent protein (GFP) expression was from Clontech. Hemagglutinin (HA)-pcDNA3 is a modified pcDNA3 vector (Invitrogen) containing a HA-tag sequence. pTrx vector encoding the thioredoxin gene [24] was from Dr S. Ishii. Histone H1 and dephosphorylated casein were from Sigma. Purified myelin basic protein (MBP), GST-c-Jun and ATF2 were described previously [25]. Acid phosphatase was from Boehringer Mannheim. All of the plasmids and vectors were prepared and purified by anion-exchange columns (QIAGEN). COS7 cells and NIH-3T3 cells were maintained in DMEM supplemented with 10% fetal calf serum and 10% calf serum, respectively.

2.2. Antibodies

Antibodies specific for HA-tag, ERK1/2 and dually phosphorylated MAPK were obtained from Boehringer Mannheim, Santa Cruz and Promega, respectively. Rabbit anti-MAPK (*Xenopus*) and anti-MAPKK (*Xenopus*) antibodies were described previously [16,26]. Horseradish peroxidase-conjugated secondary antibodies for Western blotting were from Amersham.

2.3. Construction of the fusion protein

A cDNA encoding *Xenopus* MAPK was digested by *Msp*I (Toyobo) and filled by T4 polymerase (Takara). A cDNA encoding *Xenopus* MAPKK was cut with *Afl*II (Takara) and then, the protruded

end was digested by Mung bean nuclease (Takara) to obtain an in frame blunt-end fragment. These DNA fragments were ligated, introduced into *Escherichia coli* hosts and the plasmid encoding a single fusion protein was recovered. The entire open reading frame was then excised from the plasmid with *Sma*I and *Not*I and inserted into the multi cloning site of pGEX-6P for expression of the GST-tagged fusion protein in *E. coli*. The coding region was also inserted into HA-pcDNA3 to obtain a mammalian expression vector of HA-tagged fusion protein.

2.4. Expression and purification of recombinant fusion protein

E. coli strain AD494(DE3) (Novagen), a thioredoxin reductase (trxB) deficient strain of BL21(DE3), was first transformed with pTrx (thioredoxin) and competent cells obtained were further transformed with the plasmid encoding the GST-tagged fusion protein. After reaching an appropriate density ($OD_{600} = 0.6$), cultures were supplemented with 1 mM isopropyl β-D-thiogalactopyranoside and further shaken for 24 h at 20°C. The *E. coli* cells were washed two times with phosphate-buffered saline (PBS) and ruptured in PBS (supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 TIU/ml aprotinin and 10 μg/ml leupeptin) by two times freeze-thawing and then sonication. The resulted extract was supplemented with final 1% Triton X-100 and clarified by a centrifugation ($17\,000 \times g$ for 60 min at 4°C). The extract was mixed with glutathione-Sepharose (Pharmacia) for 3 h at 4°C and the beads were washed extensively with PBS containing 1% Triton X-100. The beads were then treated overnight with PreScission protease (40 U/ml) in a cleavage buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT), pH 7.0) and the fusion protein without GST-tag was collected by centrifugation. The crude fusion protein was further purified by column chromatography using a ResourceQ column (0–1000 mM NaCl gradient in a buffer containing 40 mM Tris-Cl, 1 mM DTT and 1 mM EDTA, pH 7.4) and dialyzed in the same buffer containing 150 mM NaCl.

2.5. Expression of tagged fusion proteins in mammalian cells

HA-pcDNA3 containing the open reading frame of MAPKK*-MAPK (see Section 3) was introduced into COS7 cells by electroporation (Bio-Rad, 230 V/960 μF, 0.4 cm gap). After 2 days, cells were washed with PBS and solubilized in 400 μl per dish of IPW buffer (50 mM Tris-Cl, 50 mM NaCl, 100 mM NaF, 10% glycerol, 2 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM dithiothreitol and 1% Nonidet-P40, pH 8.0), supplemented with 0.1 TIU/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF. The lysates were then centrifuged at $17\,000 \times g$ for 60 min at 2°C and supernatants were recovered as cell extracts. NIH-3T3 cells were transfected with the mammalian expression vector described above, 1 day after plated using LipofectoAMINE PLUS Reagents (Gibco) in serum-free Opti-MEM medium. After 3 h exposure to DNA, medium was changed to DMEM+10% calf serum and further cultured for 24 h before microscopic examination.

2.6. Immunoprecipitation

The equal amounts of cell extracts (1 mg proteins) were precleared by mixing with non-immune serum (2 μl), protein G-Sepharose (Pharmacia, 15 μl) and OmniSorb suspension (Calbiochem, 300 μl). The precleared lysates were mixed with antibodies against HA-tag (4 μg) and rotated overnight at 4°C. 15 μl of the protein G-Sepharose was added and further incubated at 4°C for 90 min. The antibody-bound beads were washed with IPW buffer for five times and used for the in vitro protein kinase assay.

2.7. In vitro protein kinase assay

10 μl of the immunoprecipitates or 1 μg of the purified kinase was incubated in a kinase assay mixture at 30°C for 60 min with continuous mixing (total volume of 30 μl). The final composition of the kinase assay mixture was 50 mM Tris-Cl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 0.03 mM (0.1 MBq) ATP and 20 μg of a protein substrate, pH 7.4. To stop the reaction, 10 μl of the sodium dodecyl sulfate (SDS) sample buffer was added and

mixtures were boiled for 5 min. The phosphorylation of the proteins was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

2.8. Phosphatase treatment

0.1 μg of purified fusion protein was mixed with 0.04 U (~0.7 μg) of potato acid phosphatase in an acidic buffer (50 mM ammonium acetate, 10 mM $MgCl_2$ and 20% glycerol, pH 5.5) and incubated for 60 min at 30°C. An aliquot was removed for analysis and one fifth volume of a high pH buffer (100 mM Tris-Cl, 100 mM $MgCl_2$ and 1 mM DTT, pH 7.5) was added, then, the mixture was further incubated 15 min at 30°C with 0.2 mM (final) of ATP in the presence of phosphatase inhibitors (1 μM sodium orthovanadate and 1 μM okadaic acid).

3. Results

3.1. Strategy to make constitutively active MAPK

The strategy to construct constitutively active MAPK is schematically illustrated in Fig. 1. We

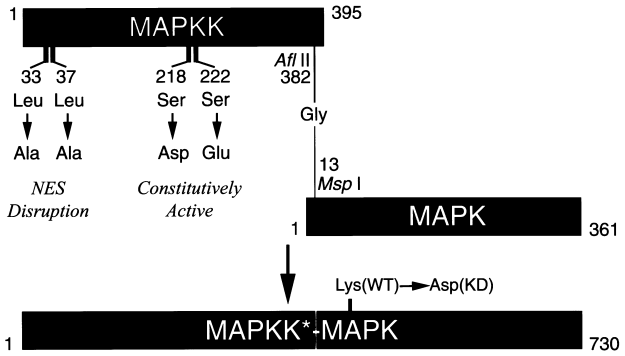

Fig. 1. Schematic illustration of the constitutively activated MAPK engineering. Ser-218 and Ser-222 of *Xenopus* MAPKK were changed to Asp and Glu, respectively, to make MAPKK constitutively active. Leu-33 and Leu-37 were mutated to Ala to disrupt a NES in MAPKK. An obtained MAPKK mutant (MAPKK*) was fused in frame to a *Xenopus* MAPK fragment. The fusion protein (MAPKK*-MAPK(WT)) is a single polypeptide containing MAPKK* at the N-terminal and MAPK at the C-terminus. Very short segments from both MAPKK and MAPK were removed during the construction as illustrated. As a negative control, the lysine residue essential for MAPK activity was mutated into Asp (MAPKK*-MAPK(KD)).

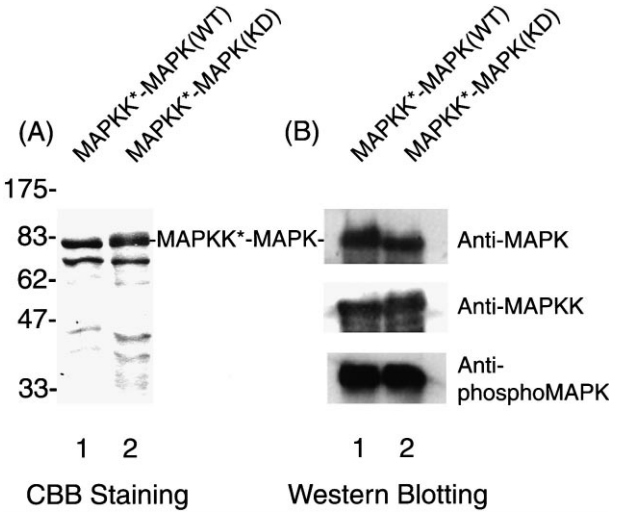

Fig. 2. Spontaneous MAPK activating phosphorylation of MAPKK*-MAPK in *E. coli*. (A) CBB staining of partially purified MAPKK*-MAPK fusion protein expressed in *E. coli*. (B) Reactivity of MAPKK*-MAPK fusion protein with anti-MAPK (top), anti-MAPKK (middle) or anti-dually phosphorylated MAPK (anti-phosphoMAPK, bottom) antibodies. Lane 1, MAPKK*-MAPK(WT); lane 2, MAPKK*-MAPK(KD).

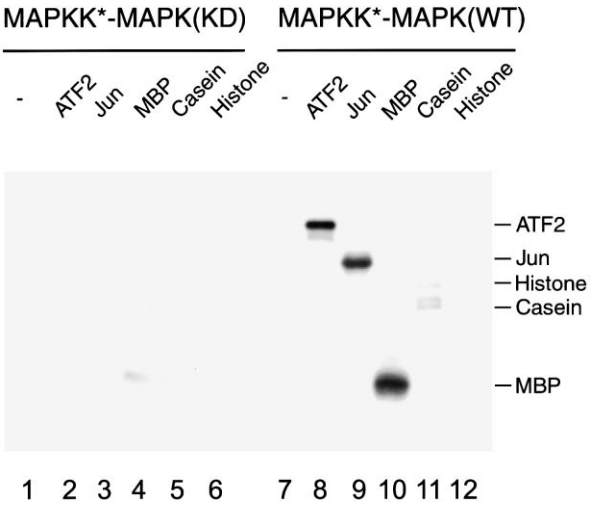

Fig. 3. Constitutive MAPK activity of MAPKK*-MAPK expressed in *E. coli*. MAPKK*-MAPK(KD) (left, lanes 1–6) or MAPKK*-MAPK(WT) (right, lanes 7–12) fusion protein was expressed in *E. coli* and purified. The fusion proteins were incubated with various indicated substrate proteins in the presence of radio-labelled ATP and the autoradiogram of SDS-PAGE was shown. The substrates used were ATF2 (lanes 2 and 8), GST-c-Jun (lanes 3 and 9), MBP (lanes 4 and 10), casein (lanes 5 and 11) and histone (lanes 6 and 12). Phosphorylation without exogenous substrate was also performed (lanes 1 and 7).

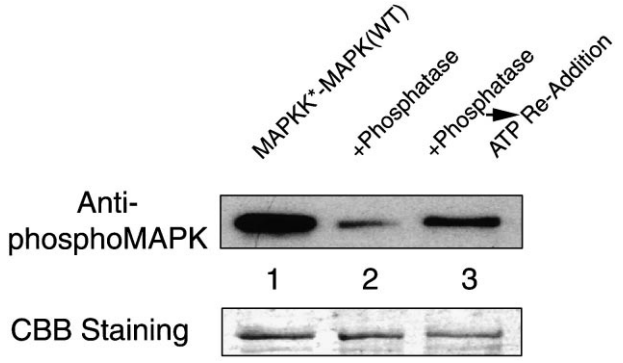

Fig. 4. Effect of dephosphorylation and re-phosphorylation on the state of the MAPK moiety of MAPKK*-MAPK(WT). Partially purified bacterially expressed MAPKK*-MAPK(WT) was probed with antibody specific for dually phosphorylated active MAPK (anti-phosphoMAPK). Lane 1, MAPKK*-MAPK(WT) without treatment; lane 2, MAPKK*-MAPK(WT) was treated with acid phosphatase; lane 3, after acid phosphatase treatment, ATP was added in the presence of phosphatase inhibitors. A lower panel indicates amounts of the fusion protein shown by CBB staining.

have previously described a constitutively active, NES-disrupted MAPKK whose kinase activity is strongest among various MAPKK mutants [23,27]. From the entire open reading frame (395 amino acids) of this mutant MAPKK, we cut out a fragment (amino acid 1–382) which is denoted as 'MAPKK*' hereafter. This fragment was fused in frame to the 5′ end of a cDNA fragment encoding *Xenopus* MAPK that lacks the N-terminal 12 amino acids. The resulted fusion DNA encodes a single polypeptide (MAPKK*-MAPK(WT)) with 730 amino acids containing constitutively active MAPKK at its N-terminal half and wild-type MAPK at its C-terminal half (Fig. 1). The lysine residue essential for kinase activity of MAPK (lysine 57 in the original MAPK subdomain II) was mutated into Asp to obtain MAPKK*-MAPK(KD), a kinase deficient negative control (Fig. 1).

3.2. Constitutive activity of the recombinant fusion protein in vitro

First, MAPKK*-MAPK was expressed as GST-tagged protein in *E. coli*. Among various conditions tested, we found that co-transformation with a thioredoxin expression vector [24] in a thioredoxin reductase deficient host was optimal for the production and recovery of the fusion protein. Lower temperatures were preferable and we cultured *E. coli* at

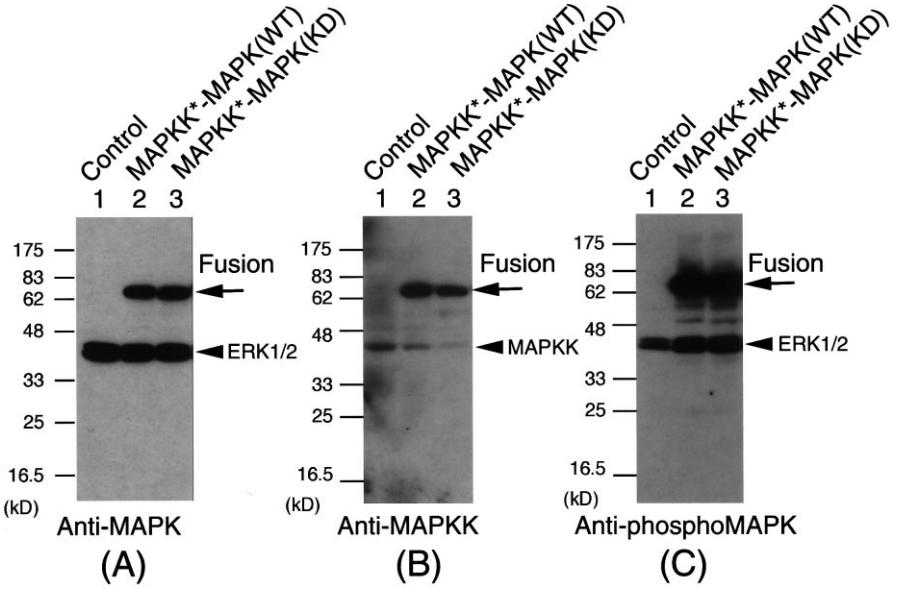

Fig. 5. Spontaneous MAPK activating phosphorylation of MAPKK*-MAPK expressed in COS7 cells. MAPKK*-MAPK(WT) (lane 2) or MAPKK*-MAPK(KD) (lane 3) was expressed in COS7 cells and the total cell lysates were probed with antibodies specific for (A) MAPK, (B) MAPKK, (C) dually phosphorylated MAPK (phosphoMAPK). Positions of endogenous MAPK (ERK1/2) and MAPKK were indicated by arrowheads and the positions of the fusion proteins were indicated by arrows. The control COS7 lysates without transfection were also examined (lane 1).

20°C after induction. The expression was readily detectable by Coomassie brilliant blue (CBB) staining of total *E. coli* lysates as a 110 kDa protein band (including GST-tag) as expected from its DNA size (data not shown). The recombinant MAPKK*-MAPK fusion protein was purified from *E. coli* lysates as described under Section 2. The purified fusion protein was observed as an 80 kDa major band (without GST-tag) with a slightly smaller proteolytic fragment (Fig. 2A). This 80 kDa polypeptide was recognized by both anti-MAPK antibody (Fig. 2B, top) and anti-MAPKK antibody (Fig. 2B, middle), confirming that the protein contained both MAPK and MAPKK moieties. Interestingly, the recombinant fusion protein was strongly reacted with an antibody that specifically recognizes doubly phosphorylated active MAPK (Fig. 2B, bottom). This result indicated that the fusion protein was doubly phosphorylated in the activation loop of the MAPK moiety. The phosphorylation was also seen in the kinase deficient mutant of the fusion protein, MAPKK*-MAPK(KD) (lane 2), indicating that autophosphorylating kinase activity of MAPK was not responsible for the activating phosphorylation.

The kinase activity of the MAPKK*-MAPK fusion protein was examined by incubating with various protein kinase substrates in the presence of radiolabelled ATP. The fusion protein phosphorylated known MAPK substrates tested (ATF2, c-Jun and MBP), but only very weakly phosphorylated other substrates (casein and histone) (Fig. 3, right, lanes 7–12). This result indicated that the substrate specificity of the fusion kinase was similar to that of MAPK. In contrast to MAPKK*-MAPK(WT), MAPKK*-MAPK(KD) did not show significant kinase activity towards these substrates (Fig. 3, left, lanes 1–6), indicating that the observed constitutive kinase activity was derived from the MAPK moiety of MAPKK*-MAPK. As reported previously [28], no kinase activity could be recovered from *E. coli* that expressed MAPK only (data not shown). Since no MAPKK was expressed in *E. coli*, the results in Figs. 2 and 3 suggest that the MAPKK portion of the fusion protein phosphorylated and activated the MAPK part spontaneously in *E. coli* cells.

We then performed a phosphatase treatment. As expected, phosphatase treatment greatly diminished the reactivity of purified fusion protein to anti-dually phosphorylated MAPK (Fig. 4, compare lanes 1 and 2). When we then added ATP exogenously in the presence of phosphatase inhibitors, the amount of dually phosphorylated MAPK of the fusion protein recovered significantly as revealed by Western blotting with anti-phosphoMAPK (Fig. 4, compare lanes 2 and 3). This result confirmed that the active form of the fusion protein could be spontaneously achieved by phosphorylation of the MAPK part with the MAPKK moiety.

3.3. Constitutive activity of the fusion protein in cells

Next, we expressed the MAPKK*-MAPK fusion protein in COS7 cells to see the constitutive activity in mammalian cells. As expected, the expressed fusion protein was recognized by anti-MAPK (Fig. 5A) and by anti-MAPKK (Fig. 5B, shown by arrows), only in the transfected cell lysates. The amount of

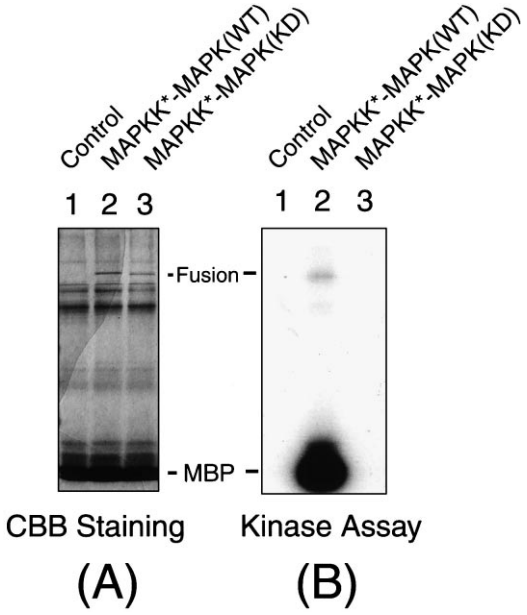

Fig. 6. Constitutive MAPK activity of MAPKK*-MAPK(WT) expressed in mammalian cells. The fusion proteins were expressed in COS7 cells by transfection and immunoprecipitated with anti-HA-tag antibody. The immunoprecipitates were incubated with [γ-^{32}P]ATP and MBP and the mixtures were analyzed by SDS-PAGE followed by CBB staining (A) or by autoradiography (B). The positions of the fusion protein and MBP were shown. Immunoprecipitates from un-transfected COS7 (lane 1), from COS7 cells transfected with MAPKK*-MAPK(WT) (lane 2) or from COS7 cells transfected with MAPKK*-MAPK(KD) (lane 3) are shown.

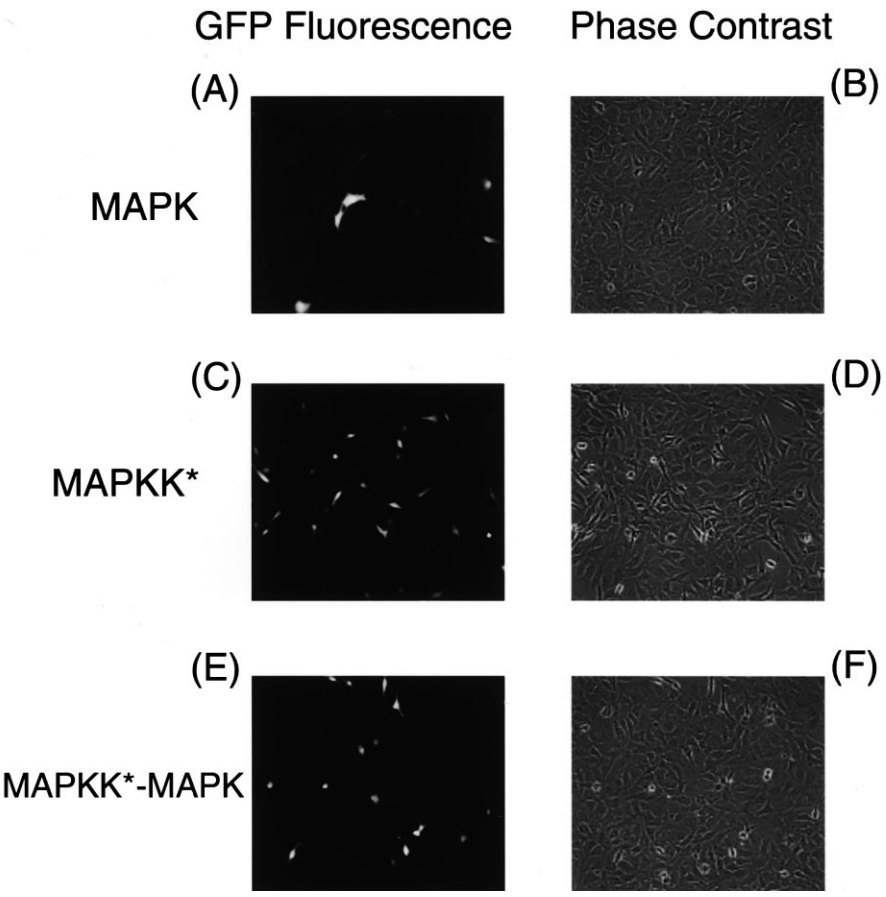

Fig. 7. Morphological transformation by the fusion protein in NIH-3T3 cells. NIH-3T3 cells were transfected with expression vectors encoding the indicated proteins along with GFP-expressing vector as a transfection marker. The cells were observed by a fluorescence microscope to see expressed GFP to identify transfected cells (left) or by a phase contrast microscope (right) to see the cell morphology. Cells transfected with MAPK (A and B), MAPKK* (C and D) or MAPKK*-MAPK(WT) (E and F) are shown.

expressed fusion protein was comparable to that of endogenous MAPK (ERK1/2) and was higher than that of endogenous MAPKK. Importantly, the expressed fusion protein was far more strongly stained than endogenous MAPK by the antibody specific for doubly phosphorylated active MAPK (Fig. 5C), suggesting the spontaneous (probably intramolecular) phosphorylation of the fusion protein at the MAPK activating site in mammalian cells. MAPKK*-MAPK(KD) was also heavily stained with the anti-phosphoMAPK (lane 3). This result again indicates that MAPK autophosphorylation was not responsible for the phosphorylation and activation of the fusion protein. The increase of the phosphorylated (i.e. activated) forms of endogenous MAPKs was minimum (Fig. 5C), showing that the MAPKK portion of the expressed fusion protein did not significantly induce the activation of endogenous MAPK. In other words, the constitutively active MAPKK in the fusion protein appears to phosphorylate the MAPK moiety of the same molecule much more efficiently than endogenous MAPK. Thus, we can assume that the effect of the expression of the fusion protein could be mainly attributable to the constitutive MAPK activity, but not to MAPKK activity, of the fusion protein.

The MAPK activity of the fusion protein was examined in vitro using MBP as a substrate after immunoprecipitation with anti-HA-tag antibody. As shown in Fig. 6B the immunoprecipitate from cells transfected with MAPKK*-MAPK(WT) exhibited strong constitutive MAPK activity. In contrast, the immunoprecipitate from cells transfected with MAPKK*-MAPK(KD) or from control un-trans-

fected cells showed no MAPK activity (Fig. 6B, lanes 1 and 3). Almost equal amounts of immunoprecipitated fusion proteins were visible by CBB staining for both MAPKK*-MAPK(WT) and MAPKK*-MAPK(KD) (Fig. 6A). Taken together, we concluded that the MAPKK*-MAPK fusion protein underwent spontaneous self-phosphorylation (MAPKK* phosphorylates MAPK in the fusion protein) in vivo and was constitutively active as MAPK.

3.4. Morphological changes induced by the fusion protein

We have been interested in the effect of expression of the constitutive MAPK activity in mammalian cells. Expression vectors were mixed with a GFP vector as a transfection marker and introduced in NIH-3T3 cells. The transfected cells could be identified by GFP fluorescence (Fig. 7, left). Cell morphology was examined under the phase contrast microscope (Fig. 7, right). NIH-3T3 cells that expressed only MAPK showed a normal flat phenotype as seen in Fig. 7A and B. When transfected with MAPKK* alone, a portion of the expressed cells became rounded, but about a half of the expressed cells remained as relatively normal spindle-like shapes under the conditions (Fig. 7C and D). This effect could be attributed to the limited activation of endogenous MAPK by MAPKK* expression (see Fig. 5C). Interestingly, almost all of the cells that expressed MAPKK*-MAPK(WT) exhibited a completely rounded transformed morphology (Fig. 7E and F). No change of morphology could be observed when the kinase deficient version of the fusion protein, MAPKK*-MAPK(KD), was expressed (data not shown). As expected by the NES disruptive mutation, most of the expressed fusion protein was distributed within the nucleus as well as cytoplasm when revealed by anti-HA staining (data not shown). Taken together, we concluded that the fusion protein possesses the constitutive MAPK activity when expressed in mammalian cells.

4. Discussion

All of the above results clearly show that the fusion protein between constitutively active MAPKK and wild-type MAPK can work as constitutively active MAPK. This constitutive activation could be observed both in *E. coli* and in mammalian cells. Although the precise mechanism of the spontaneous activation remains to be clarified, it is not unlikely to postulate intramolecular phosphorylation of the MAPK moiety by the MAPKK portion of the fusion protein. This mechanism should ensure the specificity and enhance the efficiency of activation, avoiding possible cross-talks between several similar MAPKK-MAPK pathways. It would be worth producing fusion proteins between other members of the MAPK family and corresponding constitutively active MAPKKs.

During the course of this study, Robinson et al. reported a similar approach to make a constitutively active MAPK [22]. They made a fusion protein between ERK2 and a nuclear localizing mutant of MEK1 and observed the constitutive MAPK activity of the fusion protein [22]. There are several differences between their approach and our strategy described here: (i) they constructed a fusion with MAPK at the N-terminal half while we put MAPKK at the N-terminal, (ii) they used an NES-disrupted, non-constitutively active MAPKK (whose activity is low), but we used an NES-disrupted, constitutively active MAPKK (whose activity is much stronger), (iii) they inserted an intervening Glu-Gly linker while we directly conjugated MAPKK with MAPK, (iv) our fusion protein can be produced in *E. coli* as a recombinant active kinase. Despite these differences, the major results obtained were similar. It is difficult to directly compare the level of the constitutive activity between these two different systems. One of the advantages of our strategy is that the fusion protein can be effectively and spontaneously activated when expressed and purified in *E. coli*. Until now, activated MAPK has only been available by phosphorylating purified MAPK with isolated MAPKK in vitro [28,29] or by co-expressing both MAPKK and MAPK separately in cells [30,31]. It will be an advantage for biochemical use to have a large amount of recombinant constitutively active MAPK from a single plasmid. In addition, the bacterial host that possesses constitutive MAPK activity can be used for other purposes such as screening of inhibitors.

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